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BRCA1 alternative splicing in breast tumorogenesis

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ABSTRACT

Introduction: Alternative splicing helps transcription process to produce many proteins from single gene and has important roles on tumorogenesis. The BRCA1 gene has almost 11 alternate spliced variants which have been mentioned as susceptibility factors for initiation and progression of breast cancer. Materials and Methods: The expression of BRCA1 was analyzed by qualitative and quantitative RT-PCR assays, among 27 samples from patients with primary breast cancer. Then the variants Δ2, Δ11b, and Δ14 were detected. Results: The BRCA1 gene found to be expressed among 85.2% of tumors. The distribution of Δ2, Δ11b, and Δ14 variants were 91.3%, 73.9%, and 78.3%, respectively. Discussion: The findings about three investigated variants emphasize on importance of BRCA1 alternative splicing for breast tumorogenesis.

Key words: BRCA1, Breast cancer, alternative splicing

INTRODUCTION

Breast cancer is one of the most common diseases affecting women worldwide. Its overall mortality each year is 519,000 deaths [1].Previous investigation studied on Iranian population showed 17 per 100,000 woman years with the peak incidence rate in the age group 45-54 years [2].Iranian patients seems to be relatively about 10 years younger than other countries [3], indicating more effective role of genetic than environmental factors and may lead to study on germ-line and somatic alterations in cancer-related genes.

In human and other higher eukaryotes, alternative splicing is a process to produce many proteins with different but related function from a single gene.

In tumorogenesis processes, the changes occurred in tumor suppressor genes, oncogenes, and genes encoding the proteins which their function promote the development of tumors, could resulted in progression of the process.

Therefore, identification of tumor- and tissue- specific spliced forms of mRNAs could confer the diagnostic factors which are suitable for prognosis and therapeutic interventions [4].

The Breast Cancer 1 (BRCA1) gene was cloned in 1994, by Miki et al [5].Mutation screening in its 22 coding exons revealed its accounting for ˜ 45% of hereditary breast cancers and ˜ 80% of families with both breast and ovarian cancers [6,7,8].In spite of finding rare mutations in sporadic cases, reduced expression and finding splice variants of its mRNA, suggest more important role for this protein in breast and/or ovarian carcinogenesis.

Among the identified BRCA1 spliced isoforms, the BRCA1-Δ11b was more attractive on the previous studies. The BRCA1-Δ11b variant was discovered by

Wilson et. al. [9]They described this variant with missing exon 4 and most of exon 11 (position 908 to 4215). The BRCA1-Δ11b protein sequence is differed from the BRCA1 GenBank sequence at two positions including a three nucleotide deletion resulting in the loss of an alanine at position 1453 and a serine to glycine substitution at amino acid position 1612.

In the current investigation, three BRCA1 isoforms containing Δ11b (includes deletion occurred on exon 11), Δ2, and Δ14 were studied and their relation with tumorogenesis process and some histopathologic data was analyzed.

MATERIALS AND METHODS

Patients

Institutional guidelines including ethical approval and informed content were considered. The tumor samples from 27 patients affected with primary invasive breast cancer have been collected from Day Hospital, Tehran. The samples were rapidly preserved in liquid nitrogen immediately after surgical excision and then were stored at -80°C.

Expression analysis

Total RNA was isolated using Trizol reagent (Invitrogen Co.). The accuracy of RNA isolation procedure was confirmed by electrophoresis. Two microgram of total RNA was used to create cDNA, by random hexamer and M-MuLV reverse transcriptase enzyme (Fermentas Co., Canada).

For validation of cDNA synthesis, amplification of β-actin's cDNA according to the previously described primers was used.

Qualitative assay

In routine RT-PCR, detection of RNAs in samples containing slight amounts of extracted RNA or cells with low expression levels could be failed by leading to the false-negative finding. Using nested PCR strategy is one of the ways to approach the improved detection.

Designing the primers on the exons, could lead to lose the false-positives results developed by DNA contamination. With this strategy, amplified fragments created by DNA samples, have longer lengths rather than corresponded cDNAs. The primers for amplification of the noted fragments were designed by the Genefisher 2 online software [10], and were listed on table 1.

PCR conditions for amplifying exons 2 and 11b was 3 min at 95°C, followed by

30 cycles containing denaturation by 94°C for 30 sec., annealing by 58°C for 45

sec., and extension by 72°C for 30 seconds and eventually final extension by 3 minutes at 72°C. This condition for amplifying exon 14 was the same with one exception in annealing temperature which was 54°C.

Diluted first round amplification products (1/10) were used as template for nested PCR. The amplified fragments lengths containing alternative variants were noted on table 2.
After distinguishing different variants on 1% agarose gel electrophoresis, sequencing of the amplified fragments confirmed the accuracy of detection.

Quantitative assay

For measuring BRCA1 expression level in tumor samples, primers and probe designed by Primer Express software version 3.0 (Applied Biosystems) were used on ABI 7500 system. Relative gene expression quantification was

calculated according to the comparative Ct method using GAPDH with previously reported conditions [11]as an endogenous control and RNaseP (Applied Biosystems) as an RNA control.

RESULTS

All of the 27 patients with primary breast cancer were female with average age of 47.15 Yrs. (31-71; std.=± 11.05) with invasive ductal carcinoma type. Tumor grade was 7.7%, 46.2%, and 46.2% for grade I, II, and III, respectively. The tumor stage IIA was found to be the most common among them.

Approximately 52% of patients have lymph node involvement. Progesterone and estrogen receptors were positive among 24% of patients.

Qualitative and quantitative results revealed BRCA1 expression among 85.2% (23 out of tumors).

Alternative splicing variants distribution among tumors was listed on table 3.

By analyzing the relationship between spliced variants and histopathologic data, significant associations were found only for variant E11b by considering the lymph node status and disease stage, (tables 4 and 5).

Table 1 . Primers sequences for amplifying exons 2, 11, and 14 of BRCA1 cDNA and quantitative PCR.

Detected fragments	Exon on request	Step	Sequence	Primer location
1-3	Exon2	First	Forwrad:5'-CTTGGTTTCCGTGGCAA-3' Reverse:5'-CTTCTTCAACGCGAA-3'	Exon 1 Exon 3
		Second	Forward: 5'- AAGCGCGGGAATTACAGA-3' Reverse:5'-TTCAACGCGAAGAGCAGA-3'	Exon 1 Exon 3
10-12	Exon 11	First	Forwrad:5'-CAAATCACCCCTCAAGGA-3' Reverse:5'-CTGAGAGGATAGCCCTGA-3'	Exon 10 Exon 12
		Second	Forwrad:5'-TCACCCCTCAAGGAACCA-3' Reverse:5'-AGAGGATAGCCCTGAGCA-3'	Exon 10 Exon 12
12-15	Exon 14	First	Forwrad:5'-AGCATCTGGGTGTGAGA-3' Reverse: 5'-CTATCTGCAGACACCTCA-3'	Exon 12 Exon 15
		Second	Forwrad:5'-GAAACAAGCGTCTCTGA-3' Reverse:5'-CCTCAAACTTGTCAGCA-3'	Exon 12 Exon 15
Quantization assay	BRCA1	Forward Reverse	5'-GTCTCCACAAAGTGTGACCACATAT-3' 5'-AGGCCCTTTCTTCTGGTTGAG-3'	Exon4 Exon5
		Probe	5'-FAM-CAAATTTTGCATGCTGAAA-TAMRA-3'	Exon4 Exon5
	GAPDH	Forward Reverse Probe	5'-GAAGGTGAAGGTCGGAGT-3' 5'-GAAGATGGTGATGGGATTTC-3' 5'-FAM-CAAGCTTCCCGTTCCAGCC-TAMRA-3'	

Table 2. PCR products lengths among different fragments

Fragments		1st round PCR	2nd round PCR	DNA contamination*
Exon 2	Δ2	217 bp	212 bp	994bp
	+2	595 bp	569 bp	1372bp
Exon 11	Δ11b	240 bp	233 bp	~1kb
	Complete exon 11	~4kb	~4kb	~5kb
Exon 14	Δ14	345bp	313bp	~14kb
	+14	411 bp	379 bp	~14kb

**PCR product bands created by amplification of DNA molecules which could not been completely removed during the RNA isolation procedure.*

Table 3. Spliced variants on exons 2, 11, and 14 among BRCA1 expressed tumors (85.2% of patients)

Variant	Δ2	+exon 2	Δ11b	Complete exon 11	Δ14	+exon 14
Frequency	91.3%	8.7%	73.9%	26.1%	78.3%	21.7%

Table 4. Association between E11b spliced variant and stage of disease.

		Stage of disease				
		I	IIA	IIB	IIIA	IIIB
Exon 11	Δ 11b	27.8%	27.8%	5%	0	5%
	Complete	0	5%	22.2%	5%	0

P= 0.03

Table 5. Association between spliced E11b and lymph node involvement.

		Lymph node involvement	
		Negative	Positive
Exon 11	Δ11b	44.5%	29.6%
	Complete exon	3.7%	22.2%

P= 0.04

DISCUSSION

Among the well-known predisposing markers for breast cancer, germline mutations in BRCA1 gene are valuable. After the cloning of the BRCA1 gene and distinguishing its coding sequence, the analyzing its sequential changes has been emphasized. So far, more than 1,000 mutations in this gene have been identified. Most of them are associated with an increased risk of breast cancer.

The BRCA1 as a tumor suppressor gene is involved in repairing damaged DNA.

In the case of damaged BRCA1, damaged DNA could not be repaired properly and so, cells containing damaged DNA cannot be destroyed. Therefore, risks for breast cancer will be increased.

In addition to mutations occurred at coding sequences, alteration on BRCA1 expression status have significant roles on accuracy of cellular proliferation. As the noted defects accumulated, cells allowed dividing uncontrollably and forming tumors. For highlighting the importance of BRCA1 expression level at tumorogenesis, many investigations were carried out [12,13,14].Alternative splicing is an essential mechanism for generating proteins with different functions from a single gene. Alternative splicing affects an estimated

70% of human genes [15,16].LKB1, KIT, KLF6, HAS1, and BRCA1 are among the investigated genes suffering this mechanism [17]. Lu, et al. were among the first investigators on BRCA1 alternative who described its variants [18].In the present study, the variants included transcripts lacking exons 2, 11b, and 14. The findings emphasize on the importance of BRCA1 alternative splicing for breast tumorogenesis. Among them, the variant Δ11b seems to have more attractive roles. More investigations on this variant might lead to emerge therapeutic and diagnostic factors on breast cancer.

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